

Control of Ras cycling by Ca^{2+}

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Abstract Ras GTPases are binary switches, cycling between an inactive GDP-bound form and an active GTP-bound form at the membrane. They transduce signals into the cytoplasm via effector pathways that regulate cell growth, differentiation and apoptosis. Ras activation is enhanced by guanine nucleotide exchange factors (GEFs); deactivation is accelerated by GTPase-activating proteins (GAPs). Recently, new roles for Ca^{2+} and diacylglycerol (DAG) in the control of Ras cycling have emerged with the discovery of a series of novel GEFs and GAPs. These regulators of Ras cycling are likely to play a key role in the information processing of Ca^{2+} and DAG signals.

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1. Introduction

Three human *ras* genes encode four proteins (H-Ras, N-Ras, K-Ras4A and K-Ras4B) that operate as binary molecular switches, cycling between an inactive GDP-bound form and an active GTP-bound form at the membrane [1]. They transduce signals from cell surface receptors into the cytoplasm via specific effector pathways that regulate cell growth, differentiation and apoptosis [2–5]. In vitro Ras exhibits slow rates of GDP/GTP exchange and GTP hydrolysis, thus the relative fraction of cellular Ras in an active conformation depends on the rates of these reactions [6]. Guanine nucleotide

exchange factors (GEFs) bind to Ras and markedly accelerate the rate of GDP dissociation. In contrast, deactivation requires the binding of GTPase-activating proteins (GAPs) that significantly enhance the intrinsic Ras GTPase activity. Overall the spatio-temporal regulation of GEFs and GAPs coordinates Ras signalling events (Fig. 1). This integration of Ras activation/deactivation is highly complex as there are multiple Ras GEFs and Ras GAPs with variable tissue-specific expression that are stimulated or inhibited – depending on the nature of the signal.

Understanding how Ras activity is controlled during cell stimulation is crucial considering the role oncogenic Ras plays in tumorigenesis [5]. Mutant Ras proteins with specific amino acid substitutions that render the GTPase insensitive to GAP stimulation are locked in the GTP-bound state resulting in aberrant downstream signalling. Thus, oncogenic Ras is constitutively active in transforming some (but not all [7]) mammalian cells and this is consistent with the fact that around 30% of human tumours contain activating Ras mutations [8].

Many receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) are linked to the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC) to generate inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates the release of the ubiquitous second messenger Ca^{2+} from internal stores to regulate a diverse set of cellular processes, with the versatility of calcium signalling relying on the remarkable number of different mechanisms for storage, release, propagation and removal of intracellular ions. This results in the spatio-temporal patterning of a Ca^{2+} response [9]. DAG production is classically associated with the mitogenic actions of protein kinase C (PKC) [10], however new targets for DAG have recently been discovered including a specific class of Ras GEFs [11,12]. Ca^{2+} itself is known to play a key role in cell proliferation and the regulation of the cell cycle [13], and growth requires a functional intracellular Ca^{2+} store [14]. Historically, there is a fundamental link between cell growth and a requirement for extracellular Ca^{2+} [15], which is also associated with a role for Ras. For example, in the 1970s chemical and viral transformation, including by Kirsten murine sarcoma virus (encoding v-K-ras) [16], led to extracellular Ca^{2+} -independent proliferation of transformed cells. Furthermore, Ras transformation was also shown to block Ca^{2+} -induced terminal differentiation of mouse epidermal keratinocytes [17,18]. This review concentrates on recent findings that provide new evidence for direct and dynamic Ca^{2+} regulation of Ras cycling by GEFs, GAPs and other control mechanisms.

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Abbreviations: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; DAG, diacylglycerol; RTK, receptor tyrosine kinase; GPCR, G protein-coupled receptor; PLC, phospholipase C; IP_3 , inositol 1,4,5-trisphosphate; GRF, guanine nucleotide-releasing factor; GRP, guanyl nucleotide-releasing protein; CalDAG-GEF, calcium and diacylglycerol-regulated guanine nucleotide exchange factor; MAPK, mitogen-activated protein kinase; CAPRI, calcium-promoted Ras inactivator; RASAL, Ras GTPase-activating-like; ERK, extracellular signal-regulated kinase; CHO, Chinese hamster ovary; PKC, protein kinase C; NFAT, nuclear factor of activated T cells

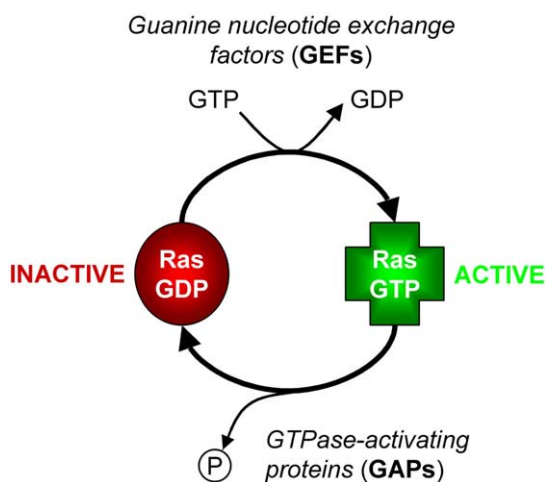


Fig. 1. The Ras GTPase cycle. The Ras activation state depends on the action of GEFs and GAPs. Oncogenic Ras is locked in the GTP-bound state and is resistant to the action of GAPs. Many Ras effectors such as Raf, RalGDS and phosphoinositide 3-kinase bind to the active GTP-bound conformation to signal downstream.

2. Ca^{2+} - and DAG-dependent Ras GEFs

2.1. Guanine nucleotide-releasing factor (GRF) family

In 1994 a seminal study by Rosen et al. showed that Ca^{2+} influx or store release activated Ras in PC12 cells and primary cortical neurones [19]. Following this finding came direct evidence for Ca^{2+} -regulated Ras activity through the discovery of a Ca^{2+} /calmodulin-dependent Ras GEF called Ras-GRF1 [20,21] (also known as CDC25^{Mm} [22]) and a closely related GEF, Ras-GRF2 [23]. The regulation and function of this family of GEFs is complex and has been recently reviewed elsewhere [12], therefore we will not discuss them in detail. GRFs are not specific GEFs for Ras; rather they are bi-functional Ras and Rac GEFs that contain both a CDC25 homology exchange domain (for Ras) and a Dbl homology exchange domain (for Rac). Multiple mechanisms for regulating GRF activation other than just by Ca^{2+} have been reported [12] so it is currently unclear exactly how, when and where these GEFs are activated to stimulate Ras or Rac signalling. Recent findings raise the possibility that Ras-GRF1 is tethered to the IB2/c-Jun amino-terminal kinase interacting protein 2 scaffold to bring the GEF, Rac and an effector, mixed lineage kinase 3, into a spatially discrete complex to stimulate the p38 signalling cascade [24]. GRF1 is highly expressed in brain tissue and the domains that mediate binding to the scaffold are also required for GEF activation by Ca^{2+} [25], which may link Ca^{2+} influx through neuronal channels to Ras/Rac signalling [24].

Mice lacking Ras-GRF1 have abnormal long-term potentiation in the amygdala suggesting that GRF1 regulation of Ras signalling has a role to play in synaptic plasticity [26]. This is an attractive hypothesis given the role of the mitogen-activated protein kinase (MAPK) pathway in the control of activity-dependent gene expression. Three independent lines of knockout mice have been generated [26–28] resulting in some dispute over the location of the learning and memory deficit [26,28]. Two lines of mice had reduced body weight and remained small during development [27,28]. This is likely to be because *grf1* is imprinted, with the finding that the Itier et al.

mice had a deficit in growth hormone production [27]. In contrast, GRF2-deficient mice are normal, and double *grf1/grf2* knockout animals are identical to the GRF1-deficient phenotype in terms of growth and development [29]. GRF1-deficient phenotypes could result from a defect in small GTPase regulation, for example by Ca^{2+} , but no study has yet demonstrated an impairment of Ras (or Rac) activation in Ras-GRF1-deficient cells.

2.2. Guanyl nucleotide-releasing protein/calcium and diacylglycerol-regulated guanine nucleotide exchange factor (GRP/CalDAG-GEF) family

This is a family of four genes encoding five Ras/Rap GEFs, viz. Ras-GRP/CalDAG-GEF II, CalDAG-GEF I, Ras-GRP2, Ras-GRP3/CalDAG-GEF III and Ras-GRP4 (see [12] for a detailed review). These GEFs appear to display some sensitivity to Ca^{2+} regulation through EF-hand domains, e.g. GRP, CalDAG-GEF I and GRP2 [30–32]. EF-hand domains are present in many Ca^{2+} -binding proteins and consist of a short Ca^{2+} -binding loop flanked on both sides by helical domains. The GRP/CalDAG-GEFs also contain a DAG-binding C1 domain in close proximity with the twin EF-hand motifs, although there is no evidence that Ca^{2+} and the EF-hands influence the DAG-binding activity of the C1 domain [33]. In T cells where Ras-GRP is highly expressed Ebinu et al. observed that Ras-GRP activated Ras in vivo even in the presence of Ca^{2+} chelators [34]. Other studies have used Ca^{2+} ionophores to manipulate intracellular Ca^{2+} but this treatment can also evoke the production of DAG by Ca^{2+} -dependent stimulation of PLC, therefore there is a question mark regarding the physiological role of Ca^{2+} in the activation of this family of GEFs. What is clear is that the C1 domain is required to dynamically recruit the GRPs to the plasma membrane in order to activate Ras [32,35,36], so it seems likely that DAG plays a dominant role in second messenger regulation of this gene family.

3. Ca^{2+} -dependent Ras GAPs

Over the last decade substantial anecdotal evidence has accumulated for a potential Ca^{2+} -feedback loop within Ras-MAPK signalling in a variety of different cell types [12]. The clearest evidence for the existence of Ca^{2+} -dependent Ras deactivation has come from studies of keratinocytes. Oncogenic Ras blocks Ca^{2+} -induced differentiation [17,18] and Ras is deactivated by Ca^{2+} influx in human keratinocytes [37,38]. An explanation for transient Ca^{2+} -triggered deactivation of Ras would be through the action of Ca^{2+} -dependent Ras GAPs and Medema et al. suggested a role for p120 Ras GAP because it translocates to the keratinocyte membrane in response to Ca^{2+} stimulation [39]. The mechanism for membrane recruitment by Ca^{2+} could involve GAP-associated proteins [39] or via an electrostatic interaction between the C2 domain of p120 Ras GAP and the membrane [40]. C2 domains are known to be Ca^{2+} -dependent phospholipid-binding domains in proteins such as conventional PKC and synaptotagmins.

A short, truncated version of the p120 Ras GAP C2 domain, the so-called CaLB motif, was shown to specifically bind phospholipids in a Ca^{2+} -dependent manner in vitro [40,41], although others (E.A. Nalefski, University of Colorado, personal communication) have been unable to demon-

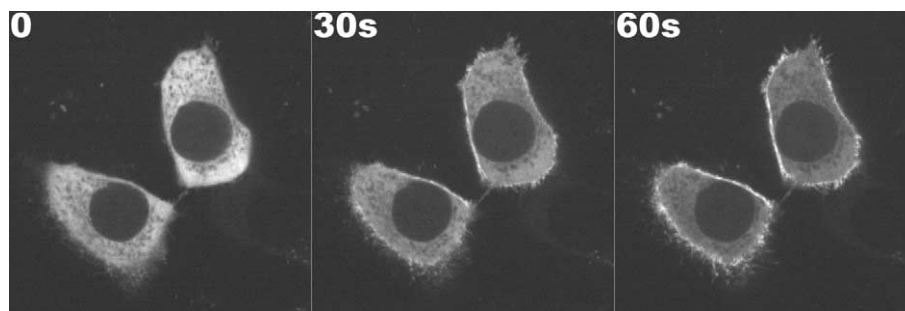


Fig. 2. Ca^{2+} -triggered translocation of CAPRI. Live HeLa cells expressing a fusion protein of green fluorescent protein (GFP) and CAPRI were imaged by rapid microscopy using a Perkin Elmer UltraVIEW system. Cells were stimulated with the cholinergic agonist carbachol to mobilise intracellular Ca^{2+} (time = seconds after stimulation). Membrane translocation induces the activation of CAPRI as a Ras GAP (unpublished and [45]).

strate such an association with natural membranes using full-length protein [42]. It is difficult to resolve p120 Ras GAP as a Ca^{2+} sensor because the C2 domain contains none of the aspartate residues known to co-ordinate Ca^{2+} ions in other Ca^{2+} -binding C2 domains [43]. Subsequently, it has also been suggested that Ca^{2+} -independent association with annexin VI might promote Ca^{2+} -triggered co-migration to the membrane [44], therefore the potential role of p120 Ras GAP in Ca^{2+} -dependent Ras regulation is still unclear. It is assumed that Ca^{2+} -induced recruitment to the membrane increases the effectiveness of the GAP by localising within the vicinity of Ras on the inner leaflet, in a similar manner to SH2 domain-dependent association with RTKs. However, a direct increase in cellular GAP activity by Ca^{2+} stimulation has yet to be convincingly attributed to endogenous or expressed p120 Ras GAP.

The prototypical Ca^{2+} -triggered Ras GAP is calcium-promoted Ras inactivator (CAPRI) [45], a member of the GAP1 family that includes GAP1^{IP4BP}, GAP1^m and Ras GTPase-activating-like (RASAL). Both CAPRI and RASAL contain a pair of C2 domains (C2A and C2B) with a full set of five putative Ca^{2+} -co-ordinating acidic residues [45]. CAPRI is basally inactive and present in the cytosol of unstimulated cells (Fig. 2). Agonist-evoked intracellular Ca^{2+} mobilisation leads to the rapid, C2 domain-dependent translocation to the plasma membrane (Fig. 2) that is paralleled by a significant reduction in cellular Ras-GTP levels in CAPRI stable Chinese hamster ovary (CHO) cell lines [45]. The CHO cells also have abrogated extracellular signal-regulated kinase (ERK) signalling downstream of GPCR stimulation but no inhibition of ERK signalling downstream of a RTK which is not coupled to PLC [45]. Although CAPRI has no detectable *in vitro* activity using standard assays, cells expressing a GAP mutant predicted to disrupt the Ras-GTP/GAP interaction have normal ERK signalling [45]. Furthermore, using a fluorescent chimera of the Ras-binding domain from Raf (GFP-RBD) and live cell imaging, we have confirmed that CAPRI is basally inactive in the cell but deactivates Ras-GTP at the plasma membrane after Ca^{2+} -triggered membrane recruitment of CAPRI (unpublished). The likely reason for CAPRI's lack of *in vitro* enhancement of the Ras GTPase reaction is a requirement for Ca^{2+} -dependent interactions with the plasma membrane, suggesting that CAPRI is locked in an inactive conformation in the cytosol until translocation is induced (Fig. 2). This hypothesis is entirely consistent with the *in vivo* data.

RASAL is highly related to CAPRI (59% identical) and is a

member of the GAP1 family that is strongly expressed in endocrine tissues [46], in contrast to CAPRI where transcript is widely expressed (data unpublished). Like CAPRI, RASAL functions as a Ca^{2+} -triggered Ras GAP, dynamically responding to Ca^{2+} signals through the regulated association with the plasma membrane (unpublished). It seems quite remarkable that cells have developed at least two different Ca^{2+} sensors tuned to detect Ca^{2+} and regulate the activation state of Ras. Currently CAPRI and RASAL are two of the most dynamically regulated GAPs for any small GTPase so far characterised. A physiological basis for having such a form of Ras regulation is unclear, yet the recent discovery of PLC ϵ as a Ras effector may go some way to providing an answer (see below).

4. Ca^{2+} /calmodulin as a brake on K-Ras activation

GEFs and GAPs regulate the Ras GTPase cycle by Ca^{2+} (and DAG). However, it is also known that Ca^{2+} /calmodulin directly regulates the activity of some Ras isoforms. This was discovered during observations that the calmodulin inhibitor W13 caused elevated Ras-GTP levels, sustained activation of ERKs and prolonged p21^{cip1} expression in low-serum NIH 3T3 cells [47]. Later it was shown that calmodulin inhibition caused preferential activation of K-Ras, and that *in vitro* K-Ras-GTP bound directly and specifically to calmodulin in a Ca^{2+} -dependent manner [48]. Stimulation of ERK and Ras signalling by calmodulin inhibition under conditions of low serum was dependent on the action of PKC, suggesting that calmodulin operates as a brake on PKC-dependent Ras activation by an undefined mechanism that could result from interfering with direct phosphorylation of K-Ras or an influence on PKC-dependent Ras GAP activity [49].

5. Perspective: is PLC ϵ the missing link?

Specific mechanisms have evolved to directly regulate Ras activity by Ca^{2+} and DAG, suggesting that Ras must have a key role in this arm of cell signalling. There are a limited number of reports in the literature that implicate oncogenic Ras in the control of Ca^{2+} oscillation frequency but the mechanisms lack detail [50,51]. Observations that Ras-transformed cells have elevated phosphoinositide metabolism have been hotly debated over the years [52] but the discovery of PLC ϵ as a new class of PLC and Ras/Rap effector [53–55] could go a long way to resolving this disputed area of Ras biology. The

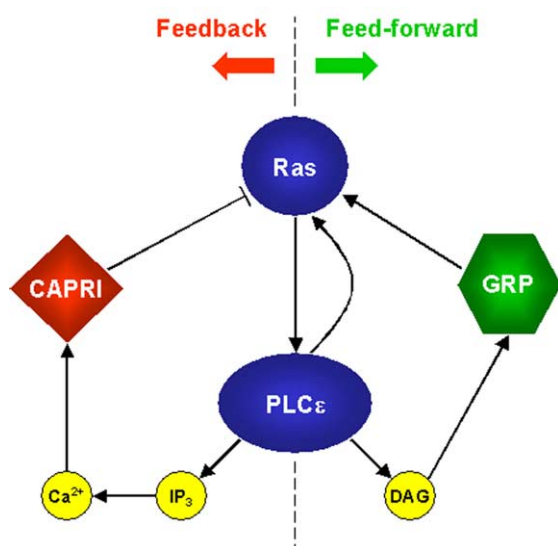


Fig. 3. Simplified scheme for the Ras-PLC ϵ signalling cassette. For clarity the roles of Rap, GRFs and calmodulin have been excluded. The ability of DAG to stimulate members of the GRP family, coupled to the intrinsic bifunctional properties of PLC ϵ , provides a mechanism for feed-forward Ras/PLC ϵ /DAG/Ca $^{2+}$ signalling. GRFs may also positively feed into this arm of the pathway by Ca $^{2+}$ stimulation, particularly in a neuronal context (see text). The nature of the feed-forward arm provides some interesting possibilities for the control of complex Ca $^{2+}$ signals, particularly repetitive Ca $^{2+}$ oscillations. Balance within this hypothetical pathway is provided by Ca $^{2+}$ -dependent negative feedback on Ras by CAPRI – virtually absent in cells transformed by oncogenic Ras and resistant to GAP activity.

existence of PLC ϵ may also provide a reason for the cellular battery of dynamic Ca $^{2+}$ - and DAG-dependent GEFs and GAPs that have recently been discovered. PLC ϵ contains both a CDC25 homology GEF domain and a pair of Ras association (RA) domains, with the RA2 domain binding Ras-GTP to stimulate enzymatic activity [55]. Yet, the regulation of PLC ϵ is highly complex since G α_{12} [54] and G $\beta\gamma$ [56] activate PLC ϵ independently of Ras, and PLC ϵ appears to operate bifunctionally as a Ras [54] or Rap1 GEF [57]. It may be that as a Ras/Rap effector, PLC ϵ will be demonstrated to play a key role in the interplay between Ras and Rap signalling and the co-ordinated generation of DAG and IP $_3$. If this proves to be the case, specificity and fidelity within this pathway can be provided by the highly dynamic spatio-temporal regulation of Ca $^{2+}$ - and DAG-regulated GEFs, GAPs and PKCs. Therefore, depending on the cellular make-up and signalling context, two major ‘products’ of PLC signalling can enable feed-forward or feedback control of the Ras/Rap-PLC signalling cassette (Fig. 3).

A long-standing issue in the Ca $^{2+}$ signalling field revolves around the cellular functions of physiological, complex Ca $^{2+}$ signals. How is information encoded by different modes of Ca $^{2+}$ signal [58]? Some of the most seductive demonstrations of Ca $^{2+}$ information processing have been at the level of gene transcription, particularly the observations that Ca $^{2+}$ signal frequency determines the efficiency of nuclear factor of activated T cells (NFAT)-induced transcription [59,60]. NFAT-driven transcription also depends on an input from Ras [61] but a role for repetitive Ca $^{2+}$ signals in the control of Ras activity has yet to be reported. There is no doubt that Ras signalling is remarkably complex with cross-talk from multi-

ple receptors and within effector pathways. Nevertheless, we propose that the binary GTPase switch is ideally suited to be a focal point for information processing by Ca $^{2+}$, particularly for decoding frequency- and amplitude-modulated Ca $^{2+}$ signals known to influence gene expression. In the future it will be interesting to see what role PLC ϵ may play in these processes. Thus, old observations and areas of dispute over the role of Ras within phosphoinositide signalling are beginning to integrate and converge, as new Ras effectors and new targets for Ras regulation by DAG and Ca $^{2+}$ are discovered.

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